PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A61K 37/02, 37/24, 37/36

A1

(11) International Publication Number: WO 93/20834

(43) International Publication Date: 28 October 1993 (28.10.93)

(21) International Application Number: PCT/US93/02874

(22) International Filing Date: 29 March 1993 (29.03.93)

(30) Priority data:
07/866,054 10 April 1992 (10.04.92) US

(71) Applicant: BRIGHAM AND WOMEN'S HOSPITAL [US/US]; 75 Francis Street, Boston, MA 02115 (US).

(72) Inventor: GONNELLA, Patricia, A. ; 77 Pond Avenue, Apt. 410C, Brookline, MA 02146 (US).

(74) Agents: FOX, Samuel, L. et al.; Sterne, Kessler, Goldstein & Fox, 1225 Connecticut Avenue, Suite 300, Washington, DC 20036-2678 (US).

(81) Designated States: AU, BG, BR, CA, CZ, FI, HU, JP, KR, NO, PL, SK, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: METHODS AND COMPOSITIONS FOR ORAL DELIVERY OF THERAPEUTIC AGENTS

(57) Abstract

The invention is drawn to methods and compositions for delivery of therapeutic agents to a subject. In particular, delivery is achieved by oral administration of a chimeric molecule having a therapeutic agent conjugated to a suitable carrier molecule wherein the carrier molecule is capable of effecting delivery of the chimeric molecule by transepithelial transport via transcytosis. The invention can obviate the need for injection of therapeutic agents unable to cross the grastrointestinal barrier.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**FR**	France	**MR**	Mauritania
AU	Australia	**GA**	Gabon	**MW**	Malawi
BB	Barbados	**GB**	United Kingdom	**NL**	Netherlands
BE	Belgium	**GN**	Guinea	**NO**	Norway
BF	Burkina Faso	**GR**	Greece	**NZ**	New Zealand
BG	Bulgaria	**HU**	Hungary	**PL**	Poland
BJ	Benin	**IE**	Ireland	**PT**	Portugal
BR	Brazil	**IT**	Italy	**RO**	Romania
CA	Canada	**JP**	Japan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SK**	Slovak Republic
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SN**	Senegal
CM	Cameroon	**LI**	Liechtenstein	**SU**	Soviet Union
CS	Czechoslovakia	**LK**	Sri Lanka	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**MC**	Monaco	**UA**	Ukraine
DK	Denmark	**MG**	Madagascar	**US**	United States of America
ES	Spain	**ML**	Mali	**VN**	Viet Nam
FI	Finland	**MN**	Mongolia		

THE INVENTION

METHODS AND COMPOSITIONS FOR ORAL DELIVERY OF THERAPEUTIC AGENTS

Field of the Invention

The invention relates to the delivery of therapeutic agents in animals, including humans.

Background of the Invention

The common routes of therapeutic agent administration are enteral (oral ingestion) and parenteral (intravenous, subcutaneous, and intramuscular) routes of administration. The intravenous route is advantageous for emergency use when very rapid increases in blood levels of the therapeutic agent are necessary. Further, the intravenous route allows for easy dosage adjustments and is useful for administration of large volumes of a drug when diluted. However, intravenous drug administration suffers from numerous limitations. One problem is the risk of adverse effects resulting from the rapid accumulation of high concentration of the therapeutic agent in plasma and/or tissues. Also, the intravenous route requires repeated injections which may cause discomfort to the subject. Further, the repeated injections may be complicated by local infections at the site of needle insertion.

Other routes of parenteral administration are painful for subjects, especially if frequent administration is required. Subcutaneous injection is sometimes used for delivery of therapeutic agents that are not irritating. However, this mode of administration is not suitable for delivering large volumes nor is it suitable for administering irritating substances which may

cause pain or necrosis at the site of injection. The intramuscular route cannot be used during anticoagulant medication and may interfere with the interpretation of certain diagnostic tests. However, it is sometimes suitable for administering therapeutic agents in moderate volumes, oily substances, and some irritating substances.

Oral administration of drugs is generally more convenient, economical, and acceptable. However, oral administration is limited where the therapeutic agent is not efficiently absorbed by the gastrointestinal tract. Absorption by the gastrointestinal tract may be inefficient for poorly soluble, slowly absorbed, or unstable therapeutic preparations. Many important therapeutic agents which need to be administered frequently are not effectively absorbed when administered orally and hence must be delivered by injection methods.

For example, compounds with molecular weights of approximately 500 daltons and above are poorly absorbed from the gastrointestinal tract. The efficacy of orally administered therapeutic agents depends, in a large part, on the agent being absorbed from the gastrointestinal tract into the circulation.

Some investigators have attempted to circumvent the above-noted problems through intranasal administration of a therapeutic agent to a subject through the use of a fusidic acid derivative adjuvant. Others have attempted to effect therapeutic agent penetration across skin through use of penetration enhancers such as chelating agents, bile salts, surfactants, acylcholines and acylcarnitines. Penetration enhancers such as Azone, oleic acid, decylmethyl sulfoxide and propylene glycol have recently been shown to promote the penetration of the anticancer drug 5-fluorouracil in hairless rat skin. Still others, noting that therapeutic agents with molecular weights of approximately 500 daltons and above are poorly absorbed from the gastrointestinal tract, suggest that drugs which are analogs to amino acids and small peptides may be absorbed by the non-passive pathway. Still other investigators attempted to effectuate

absorption from the gastrointestinal tract through use of liposome entrapped therapeutic agents. Certain investigators have claimed that intact liposomes are identified in the circulation after oral administration. However, other investigators note that experiments wherein the antibiotic gentamicin was entrapped in a liposome, it was not detected in the circulatory system of subjects.

Others have developed a system for delivering neuropharmaceutical agents to the brain by receptor-mediated transcytosis through the blood-brain barrier. Chimeric peptides were developed consisting of a transportable peptide (such as insulin, transferrin, insulin-like growth factor I, insulin-like growth factor II, basic albumin and prolactin) conjugated to hydrophilic peptide neuropharmaceutical agents which alone are not generally transported across the blood-brain barrier.

Notwithstanding the above-noted developments in the art of drug delivery, it is clear that there is a need for novel methods and compositions for oral delivery of therapeutic agents to a subject's circulatory system.

Description of Related Literature

The delivery of neuropeptides through the blood-brain barrier can be found in: Pardridge, W., United States Patent No. 4,801,575;

Liposomally entrapped insulin for gastrointestine absorption is discussed in Patel *et al.*, *Biochem. Soc. Trans.*, *5(4)*:1054-1055 (1977); Aprahamian *et al.*, *Chim. Oggi.* *3*:13-15 (1987).

Absorption enhancing agents for the gastrointestinal system can be found in: Fix, J., *Controlled Release* *6*:151-156 (1987); Sugibayashi *et al.*, *J. Pharm. Pharmacol.*, *37*:578-580 (1985); Touitou *et al.*, *Int. J. Pharm.*, *27*:89-98 (1985); and Goodman *et al.*, *J. Invest. Dermatol.*, *91*:323-327 (1988).

The absorption of drugs which are analogous to amino acids or small peptides is discussed in: Sinko *et al.*, *J. Controlled Release* 6:115-121 (1987).

Intranasal administration of therapeutic agent through the use of a fusidic acid derivative adjuvant is discussed in: Carey *et al.*, United States Patent No. 4,746,508.

Summary of the Invention

Delivery of a therapeutic agent to a subject is achieved by administering to the subject a chimeric molecule wherein the chimeric molecule comprises a therapeutic agent to be delivered conjugated to a suitable carrier wherein the carrier is capable of transport across epithelial cells via transcytosis. Delivery of therapeutic agents across epithelial cells in the liver, kidney, and gastrointestinal tract can occur by the chimeric molecules of the present invention. When the chimeric molecules of the present invention are administered orally, a therapeutic agent can be delivered to a subject's circulatory system by transcytosis of the carrier across epithelial cells of the gastrointestinal tract. Thus, the present invention obviates the need for injection of therapeutic agents unable to cross the gastrointestinal barrier.

Description of the Specific Embodiments

Methods and compositions are provided for delivery of therapeutic agents to a subject. The methods involve conjugating a therapeutic agent to be delivered to a suitable carrier wherein the carrier is capable of effectuating delivery via transcytosis. The resultant conjugate is a chimeric molecule which acts as a vehicle for delivery of the therapeutic agent. The therapeutic agent can be delivered to a subject's circulatory system when the chimeric molecules of the present invention are

administered to the subject orally. In this embodiment, the chimeric molecule is absorbed into the circulation from the gastrointestinal tract by transcytosis.

By "subject" is intended both human and non-human animal subjects who are administered the chimeric molecules of the present invention. Specifically intended are mammalian subjects. More specifically intended are human subjects.

By "therapeutic agent" is intended drugs and/or medicinal peptides useful for treating a medical or veterinary disorder, preventing a medical or veterinary disorder, or regulating the physiology of a human being or animal.

Drugs for which the method of administration of the invention is particularly important are peptides. Suitable peptides include, but are not limited to, insulin, proinsulin, glucagon, parathyroid hormone and antagonists of it, calcitonin, vasopressin, renin, prolactin, growth hormone, thyroid stimulating hormone, corticotropin, follicle stimulating hormone, luteinizing hormone, chorionic gonadotropin, atrial peptides (a natriuretic factor), interferon, tissue plasminogen activator, gamma globulin, factor VIII, and analogs and/or chemical modifications of these peptides.

The invention can also be used to administer hormone releasing hormones, e.g., growth hormones releasing hormone, corticotropin releasing factor, luteinizing hormone releasing hormone, growth hormone release inhibiting hormone (somatostatin) and thyrotropin releasing hormone.

Other suitable drugs include the physiologically active enzymes: transferases, hydrolases, isomerases, proteases, ligases, and oxidoreductases such as esterases, phosphatases, glycosidases and peptidases; enzyme inhibitors such as leupeptin, chymostatin and pepstatin; and growth factors such as tumor angiogenesis factor, epidermal growth factor, nerve growth factor and insulin-like growth factors. Other suitable drugs are those normally absorbed only to a limited extent across

the gastrointestinal mucosa after oral administration; e.g., antihistamines (e.g. diphenylhydramine and chlorpheniramine), and drugs affecting the cardiovascular (e.g., antihypertensives), renal, hepatic and immune systems (including vaccines). Additionally, sympathomimetic drugs, such as the catecholamines (e.g., epinephrine) and non-catecholamines (e.g., phenylephrine and pseudoephedrine) may be administered according to the method of the present invention.

Drugs such as anti-infective agents, including antibacterial, antiviral and antifungal agents may also be administered according to the method of the present invention. For example, antibiotics such as the aminoglycosides (e.g., streptomycin, gentamicin, kanamycin, etc.) are normally not adequately absorbed after oral administration, and may therefore be advantageously administered by the method of the invention.

Many other drugs may also be administered according to the invention, e.g., the many drugs currently used to treat arthritis such as narcotic pain relievers. Anti-inflammatory agents (e.g., indomethacin, dexamethasone and triamcinolone), anti-tumor agents (e.g., 5-fluorouracil and methotrexate) and tranquilizers such as diazepam may also be administered according to the invention.

Other suitable drugs are the water insoluble, fat-soluble hydrophobic drugs, e.g., steroids, such as progesterone, estrogens (including contraceptives such as ethinyl estradiol) and androgens and their analogs, and the fat-soluble vitamins, e.g., vitamins A, D, E and K, and their analogs.

Significant absorption of therapeutic agents from the gastrointestinal tract is a prerequisite for effective oral delivery to the circulation of a subject. As discussed above, there are numerous drugs and medicinal peptides which alone are ineffectively absorbed from the gastrointestinal tract into the circulation. Thus, it is clear that a reproducible means for oral delivery of therapeutic agents would be highly

desirable. The method of the present invention provides such a reproducible delivery system.

In the method of the present invention, growth factors having receptors in epithelial cells of the gastrointestinal tract are used as carriers to transport therapeutic agents from the gastrointestinal tract into the circulation of a subject. Thus, when these growth factors are conjugated to a therapeutic agent, the resultant conjugate is a chimeric molecule which acts as a vehicle allowing oral delivery of a therapeutic agent into the subject's circulatory system. Further, growth factor fragments or analogs can also be used as carriers for delivery of therapeutic agents to the subject. All that is required is that the growth factor fragment or analog can be conjugated to a therapeutic agent, binds the growth factor receptor and is capable of transepithelial transport via transcytosis.

By "carrier" is intended macromolecules which, when conjugated to a therapeutic agent, are capable of effecting delivery of the therapeutic agent to a subject via transcytosis. Specifically by "carrier" is intended growth factors, or fragments or analogs thereof, which bind a growth factor receptor and, when conjugated to a therapeutic agent, are capable of effectuating delivery of the therapeutic agent via transcytosis. Suitable carriers include, but are not limited to: epidermal growth factor (EGF), transforming growth factor α (TGF-α), and fragments or analogs of these growth factors wherein the fragments or analogs are capable of binding the growth factor receptors and are capable of effectuating oral delivery of a therapeutic agent to the circulation.

Mature epidermal growth factor (EGF), a 53-amino acid single chain polypeptide, has been well characterized in the art. See, for example, Gregory, H., *Nature 257*:325 (1975); Komoriya *et al., Proc. Natl. Acad. Sci. USA 81*:1351 (1984); EPA 0 326 046; UK Patent Application GB 2 172 890 A; EPA 335 400, the disclosures of which are hereby incorporated by reference. The amino acid and nucleic acid sequences of EGF are known. EGF has three disulfide bonds which define three

looped regions from residues 1-20, 14-31, and 32-53. EGF is a potent stimulator of cellular proliferation and inhibitor of gastric acid secretion. Epidermal growth factor receptors have been found in cells of the gastrointestinal tract, in hepatocytes and in kidney cells. In the gastrointestinal tract, these epidermal growth factor receptors have been shown to mediate transepithelial transport of epidermal growth factor into the circulation. This pathway is called receptor-mediated transcytosis. Transepithelial transport of epidermal growth factor can also occur by transcytosis which is not receptor-mediated. Thus, the growth factor can be absorbed from the gastrointestinal tract by either receptor mediated transcytosis or by non-specific association. *See* Gonnella, *et al., J. Clin. Invest. 80*:22-32 (1987); Gonnella, *et al., Advanced Drug Delivery Review 1*:235-248 (1987); Thompson, J., *Am. J. Physiol. 254*:G429-G435 (1988); Thornberg *et al., Am. J. Physiol. 253*:G68-G71 (1987); Weaver *et al., Gastroenterology 98*:828-837 (1990); and Pothier *et al., FEBS. Lett. 228(1)*:113-117 (1988), the disclosures of which are hereby incorporated by reference.

In addition to epithelial cells of the gastrointestinal tract, liver and kidney cells also are known to have epidermal growth factor receptors. *See*, for example, Dunn *et al., J. Cell Biol. 102*:24-36 (1986) and Brandli *et al., J. Biol. Chem. 266 (13)*:8560-8666 (1991), the disclosures of which are hereby incorporated by reference. Substantial amounts of EGF are cleared from the circulation by hepatocytes via receptor mediated endocytosis and are subsequently degraded within lysosomes. See, Dunn *et al., J. Cell Biol. 102*:24-36 (1986). Further, the EGF receptor is known to mediate uptake of EGF in kidney cells. See, Brandli *et al., J. Biol. Chem. 266(13)*:8560-8566 (1991). Thus, the present invention also provides a means for delivering therapeutic agents to the liver and the kidney.

Drug transport in the intestine, liver and kidney is similar because in each case transport occurs across a barrier of epithelial cells. If the

target for delivery is the liver or kidney, administration of the chimeric molecule may be parenteral rather than enteral.

Komoriya *et al.* provided biologically active synthetic fragments of EGF and localized a major receptor-binding region of EGF. *See*, Komoriya *et al., Proc. Natl. Acad. Sci. USA 81*:1351-1355 (1984). Synthetic peptide fragments of EGF were shown by Komoriya *et al.* to compete with intact EGF in binding to the EGF receptors. Further, the synthetic fragments of the epidermal growth factor were shown to induce a series of cellular responses like those to EGF. Thus, EGF fragments or analogs which bind to the EGF receptor can be used as carriers in the present invention.

Mature transforming growth factor α (TGF-α) is a 50-amino acid containing polypeptide sharing about 32% sequence homology with EGF. Like EGF, TGF-α induces a potent mitogenic response in cells. The amino acid and nucleic acid sequences for TGF-α are known. See, for example, Derynck *et al., Cell 38*:287-297 (1984), the disclosure of which is hereby incorporated by reference. TGF-α competes with EGF for binding to the EGF receptor and exhibits all the apparent biological activities of EGF. Tam *et al., Proc. West Pharmacol. Soc. 29*:471-474 (1986) (the disclosure of which is hereby incorporated by reference), synthesized several fragments of TGF-α to identify the active portion of this growth factor. These fragments, like intact TGF-α, bind EGF receptors which mediate all apparent biological activities of EGF. *See*, also, Tam *et al., Int. J. Pept. Protein. Res. 38(3)*:204-211 (1991) and Nestor *et al., Biochem. and Biophys. Res. Comm. 129(1)*:226-232 (1985), the disclosures of which are hereby incorporated by reference. In the present invention, intact TGF-α and TGF-α fragments and analogs which bind the EGF receptor can be used as carriers in the present invention.

In the present invention, chimeric molecules are formed by conjugating a therapeutic agent to a suitable carrier wherein the carrier is capable of transporting the conjugate across epithelial cells via

transcytosis. By the term "chimeric molecule" is intended a conjugate molecule comprising a therapeutic agent conjugated to a suitable carrier wherein the carrier is capable of transporting the conjugate across epithelial cells via transcytosis. Specifically, by "chimeric molecule" is intended a conjugate molecule comprising a therapeutic agent conjugated to a growth factor or a growth factor fragment or analog wherein the growth factor or growth factor fragment or analog is capable of effectuating delivery of the therapeutic agent to a subject via transcytosis. More specifically, by "chimeric molecule" is intended a conjugate molecule wherein a therapeutic agent is conjugated to EGF or fragments or analogs thereof or TGF-α or fragments or analogs thereof capable of effectuating delivery via transcytosis.

The compositions of this invention can be employed in mixture with conventional excipients, i.e., pharmaceutically acceptable organic or inorganic substances suitable for enteral or parenteral application which do not deleteriously react with the chimeric molecules. Suitable pharmaceutically acceptable substances include but are not limited to water, salt solutions, alcohol, vegetable oils, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, perfume oil, fatty acid monoglycerides and diglycerides, petroethral fatty acid esters, hydroxymethylcellulose, polyvinylpyrrolidone, etc. The pharmaceutical preparations can be sterilized and if desired mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavoring and/or aromatic substances and the like which do not deleteriously react with the chimeric molecules.

For parenteral application, particularly suitable are solutions, preferably oily or aqueous solutions as well as suspensions, emulsions, or implants, including suppositories. Ampules are convenient unit dosages.

For enteral application, particularly suitable are tablets, dragees or capsules having talc and/or a carbohydrate carrier binder or the like, the

carrier preferably being lactose and/or corn starch and/or potato starch. A syrup, elixir or the like can be used wherein a sweetened vehicle is employed. Sustained release compositions can be formulated including those wherein the active component is protected with differentially degradable coatings, e.g., by microencapsulation, multiple coatings, etc.

The chimeric molecules of the present invention are made by conjugating the therapeutic agent to be delivered to a suitable carrier. Since the growth factor carriers described in the present invention bind growth factor receptors, the conjugation needs to be carried out in a manner that will not prevent binding of the growth factor to the receptor. The receptor binding regions (discussed *supra*) of EGF and TGF-α are necessary and sufficient for binding to the epidermal growth factor receptor. Further, the conjugation of the carrier to the therapeutic agent must not prevent transcytosis of the resultant chimeric molecule. As discussed above, the receptor binding regions of the EGF and TGF-α are known in the art. *See*, for example, Nestor *et al., Biochem. and Biophys. Res. Comm. 129(1)*:226-232 (1985); Tam *et al., Proc. West. Pharmacol. Soc. 29*:471-474 (1986); Tam *et al., Int. J. Pept. Protein Res. 38(3)*:204-211 (1991); and Komoriya *et al., Proc. Natl. Acad. Sci. USA 81*:1351-1355 (1984). Thus, it is well within the purview of one of ordinary skill in the art using known conjugation techniques to conjugate therapeutic agents to the growth factors disclosed herein at appropriate regions on the carrier molecules.

Various conjugation techniques are known in the art. The following conjugation techniques are provided by way of illustration. Other conjugation techniques can also be used when appropriate. Where the therapeutic agent is a medicinal peptide, conjugation may be carried out using bifunctional reagents which are capable of reacting with each of the peptides (i.e., the medicinal peptide and carrier peptide) and forming a bridge between the two. One preferred method of conjugation involves peptide thiolation wherein the two peptides are treated with reagents such

as N-Succinimidyl 3-(2-pyridyldithio)propionate(SPDP) to form a disulfide bridge between the two peptides to form the chimeric molecule. Other known conjugation agents may be used, so long as they provide linkage of the two peptides (i.e., the carrier peptide and the medicinal peptide) together without denaturing them. Preferably, the linkage can easily be broken once the chimeric molecule has entered the circulation. Suitable examples of conjugation reagents include: glutaraldehyde and cystamine and EDAC. Conjugation of peptides using glutaraldehyde is described in Poznansky *et al., Science 223*:1304-1306 (1984), the disclosure of which is herein incorporated by reference. Conjugation of peptides using cystamine and EDAC is described in Ito *et al., Mol. Cell. Endocrinol. 36*:165 (1984), the disclosure of which is herein incorporated by reference.

Further, the conjugate chimeric molecules of the present invention can be in the form of a fusion peptide made by recombinant DNA techniques. Examples of producing EGF by genetic engineering techniques are provided in EPA 0 335 400, EPA 0 326 046, and GB 2 172 890. An example of producing TGF-α by genetic engineering techniques is provided in Derynck *et al., Cell 38*:287-297 (1984). Thus, one of ordinary skill can make fusion peptides suitable as chimeric molecules for delivery of therapeutic agents to a subject. The use of recombinant DNA techniques requires knowledge of the nucleic acid sequence of the medicinal peptide to be delivered. The nucleic acid fragment corresponding to the medicinal peptide is linked to a nucleic acid fragment corresponding to a carrier to form a recombinant molecule. The recombinant molecule is then operably linked to an expression vector and introduced into a host cell to enable expression of a fusion peptide useful as a chimeric molecule in the present invention. See *Molecular Cloning, A Laboratory Manual*, Sambrook *et al.*, eds. Cold Spring Harbor Laboratory, 2nd. Ed., Cold Spring Harbor, NY (1989).

As indicated above, mature EGF is a 53-amino acid single chain polypeptide. The amino acid and nucleic acid sequences of EGF are

known. Potentially, one or more of each of the 53 amino acid residues of EGF can be used as a site for conjugating the therapeutic agent. In a preferred embodiment, an EGF fragment encompassing amino acid residues 14-31 of mature EGF can be used as a carrier in the present invention. Potentially, one or more of these residues (14-31) can be used as a site for conjugating the therapeutic agent.

As indicated above, mature TGFα is a 50-amino acid polypeptide. The amino acid and nucleic acid sequences of TGFα are known. Potentially, one or more of each of the 50 amino acid residues of TGFα can be used as a site for conjugating the therapeutic agent. In a preferred embodiment, a TGFα fragment encompassing amino acid residues 34-43 of mature TGFα can be used as a carrier in the present invention. Potentially, one or more of these residues (34-43) can be used as a site for conjugating the therapeutic agent.

After a therapeutic agent has been conjugated to EGF or TGFα at one or more of the amino acid residues specified above, the suitability of the resultant conjugate for purposes of the present invention can be tested following the protocols set forth in the Experimental section of the specification.

The chimeric molecules of the present invention can be administered to subjects at a dosage range of from about 15 mg to about 75 mg. However, the dosage range may differ depending on the subject and the therapeutic agent to be delivered.

Optimal dosages can be determined by one of ordinary skill in the art using conventional techniques. As a general rule, the dosage levels should correspond to the accepted and established dosages for the particular therapeutic agent to be delivered.

The following examples are offered by way of illustration and not by way of limitation.

Experimental

1. *In Vitro* Studies

The purpose of this example is to show that TGF-α or EGF receptor-binding fragments alone and in conjugation with a foreign peptide bind to preparations of brush border membrane vesicles.

Microvillous membrane fractions (MVM) are prepared from adult rat and human small intestine by the calcium precipitation method (Schmitz *et al., BBA 323*:98-112 (1973), Kessler *et al., BBA 506*:136-154 (1978) as modified by Sterne *et al., Ped. Res. 18(12)*:1252-1257 (1984). Intestinal segments are removed from anesthetized rats or are obtained after a gastric bypass procedure (human tissue) and washed immediately with cold saline. Tissue processing is performed at 4°C according to the following method. Scrapings are homogenized in a conical binding tube in 500 mM mannitol, 10 mM hepes buffer pH 7.5. After a 1:6 dilution and filtration through fine nylon mesh (40 μm pore size), $CaCl_2$ is added to a final concentration of 10 mM. After 10 minutes and occasional mixing by inversion the homogenate is centrifuged at 2500 x g for 15 minutes.

Subsequently, the supernatant is centrifuged at 28,000 x g for 30 minutes. The pellets are resuspended in 100 mM mannitol, 10 mM Tris-HCL, pH 7.4. MVM protein concentration is determined by the method of Lowry *et al., (J. Biol. Chem. 193*:262-275 (1951)).

A receptor-binding carrier peptide is derived from the A, B or C disulfide loop of TGF-α. Synthetic peptides representing residues 8-50 (ABC loop), 8-21 (A loop), 16-32 (B loop), and 34-43 (C loop) are prepared by a stepwise solid phase method using a differential acid lability protection scheme of N^{α}-tertbutoxycarbonyl and side chain benzyl protecting groups. In particular, synthetic peptides which are the third

disulfide loop of TGFα (residues 34-43), are prepared by the stepwise solid phase method.

A receptor-binding carrier peptide from EGF is derived from the three looped regions defined by disulfide bonds. Synthetic peptides representing residues 1-53 are prepared by the stepwise solid phase method. In particular, synthetic peptides which are a cyclic fragment of EGF, containing residues 14-31, are prepared by the stepwise solid phase method.

The TGF-α or EGF carrier peptide fragments alone and in conjugation with a foreign peptide are labeled with ^{125}I using iodobeads (Pierce Chemical Co.) according to the method of Markwell (*Anal. Biochem. 125*:427-432 (1982)). Free iodine is removed by filtration on a Sephadex column followed by dialysis in phosphate buffered saline (PBS) at 4°C overnight. Labeled peptides are incubated with MVM preparations in the presence and absence of 1000 fold excess cold EGF. Specificity of binding is assessed by electron microscopic autoradiography.

Biochemical analysis of binding is performed using the miniature ultracentrifuge separation technique (Albers *et al., Anal. Biochem. 96*:395-402 (1979)). The advantages of this method for measurement of radioligand binding in aqueous medium are threefold: 1) the rapidity (30 seconds) in separating the bound from the unbound fraction, 2) the small volume (100 μl) of assay medium which permits a relatively small excess of ligand over receptor to be employed, 3) the simplicity of manipulations which allows a high degree of replication.

Airfuge tubes (5 x 20 mm; Beckman Instruments Inc., Spinco Division, Palo Alto, CA) are pretreated overnight at 4°C with a 1 mg/ml solution of cold peptide to inhibit nonspecific protein absorption from the final incubation mixture. For the incubations, radiolabeled peptide solutions between 0.01 μg and 1 mg/ml are used in Tris-HCL (30 mM pH 7.4) containing 125 mM NaCl. Specific activity of samples is determined and 100 μl of MVM suspensions varying in protein content between 0.1

and 4.0 mg/ml are mixed with the same volume of protein-label solution and incubated at 37°C, and 4°C. All incubation experiments are run in duplicate. Aliquots of the mixture (100 μl) are centrifuged for 15 minutes at 178,000 x g in a Beckman Airfuge using the A-100/30 fixed angle rotor in order to separate unbound protein from MVM. Supernatants are removed and the pellets counted. To control for nonspecific absorption duplicate airfuge tubes are incubated with the protein-label solution and buffer 1:1. Their counts are subtracted from the total counts obtained from MVM. Results are expressed as μg of ^{125}I-protein Eg/mg of MVM.

2. *In vivo* Studies

A. The purpose of the following example is to show that the EGF or TGFα fragments (described above) alone and in conjugation with a foreign peptide are transported across the epithelium and are detected in the plasma and target tissues.

Iodinated EGF or TGFα receptor-binding fragments alone and in combination with a foreign peptide (described above) are injected into ligated loops *in vivo* of proximal or distal rat intestine for time intervals of 30 minutes to 2 hours. Ligated loops are prepared by making an incision along the midline of the abdomen and exposing a segment of intestine. Ligatures are passed through the mesentery without disturbing the mesenteric circulation. The distal ligature, placed 3 cm proximal to the cecum is tightened. A 27 gauge needle is passed through the proximal ligature, the ligature is tightened around the needle and the protein-label solution is injected. As the needle is withdrawn the proximal ligature is tightened further to provide a leak free compartment. After the appropriate time interval the ligated loop is excised intact. The loop lumen is rinsed with PBS and subsequently rinsed with fixative consisting of freshly depolymerized formaldehyde, 2.5% glutaraldehyde and 4 mM

$CaCl_2$ in 0.1 M sodium cacodylate buffer, pH 7.4 Tissues are then immersed in a drop of fixative, chopped at 1 mm and processed for autoradiography.

After 2-4 hours of fixation at room temperature, tissue slices are rinsed in 0.1 M cacodylate buffer and postfixed in 1% osmium in cacodylate buffer. Next, tissues are stained en bloc in 1% uranyl acetate, dehydrated in graded ethanols and embedded in Epon-Araldite. For light microscopic autoradiography, 1 μm sections are stained with iron hematoxylin, coated with Ilford K5 emulsion (diluted 1:1 with distilled water) exposed for 1-4 weeks and developed for 4 minutes in Kodak D-19 at 18°C.

For electron microscopic (EM) autoradiography, thin sections are collected on formvar-coated nickel grids and stained with lead citrate. Grids are carbon coated, placed on a glass slide and coated with a thin film of Ilford L4 emulsion (diluted 1:4 with distilled water) by the loop method (Caro, L.G. and R.P. Vantubergen, *J. Cell Biol. 15*:173-178 (1962)). The emulsion coat consists of a monolayer of silver halide crystals as confirmed by electron microscopy of undeveloped grids. After 6-8 weeks, autoradiographs are developed in Kodak D-19 (diluted 1:9) for 45 seconds at 18°C and are fixed in 24% sodium thiosulfate for 3 minutes at 18°C. Sections are examined and photographed with a JEOL 100X electron microscope.

The distribution of silver grains in subcellular compartments and at the basolateral surface are quantitated according to the following procedure. For each time interval 15 well-oriented cells sectioned along a central longitudinal axis are selected and all grains over these cells are counted. Compartments are demarcated into microvilli, apical vesicular compartments, multivesicular bodies (lysosomes), nucleus, and basal vesicular compartments. Grains are assigned to lateral or basal membranes if they span the membrane or lay within 900 nm, the estimated half-distance (HD) for Ilford L4 emulsion exposed by ^{125}I

(Salpeter *et al., J. Cell Biol.* 76:127-145 (1978)) and processed as detailed above. Relative surface densities of cell compartments are calculated from the same section by outlining compartment boundaries overlaying a standard grid and scoring allocations of grid intersects (Weibel *et al.,* In: *Principles and techniques of electron microscopy,* M.A. Hyat, editor, Van Nostrand Reinhold, New York 239-296 (1975)). These values are used to calculate the relative grain density in each compartment (Salpeter, M. and F.A. McHenry, In: *Advanced techniques in Biological Electron Microscopy,* J.K. Koehler, editor, Springer Verlag, New York, 113-152 (1973)).

The presence of peptide in sera and tissue extracts is verified by polyacrylamide gel electrophoresis (SDS-PAGE)/autoradiography, and reversed-phase HPLC in the presence of strong acid and organic solvents.

For SDS-PAGE, samples of serum are precipitated with 20% trichloroacetic acid (TCA) washed twice in acetone and solubilized in sample buffer containing SDS and mercaptoethanol. Aliquots, containing 40,000 counts per minute (cpm) are applied to 13-22% polyacrylamide gels along with 40,000 cpm of stock protein-label. Gels are stained with Coomasie Blue, dried, autoradiographed by exposure to XAR5 film (Eastern Kodak Co., Rochester, N.Y.) at -80°C and developed after 1-7 days.

Reversed-phase HPLC is performed on a C18 Bondapack column (Waters Instruments, Inc., Rochester, MN) equilibrated in 0.1% trifluoroacetic acid (TFA) with 21% acetonitrile. Samples are diluted 1:1 with starting solvent, applied to a 7.8 x 30 cm column and the column is washed 5 minutes in the same solvent. Material is eluted with a 20 minute acetonitrile gradient (21-63% in 0.1% TFA). Fractions are collected and radioactivity in each is measured in a gamma scintillation counter (75% efficiency; Packard Instruments Co., Downers Grove, IL).

In other experiments the amount of fed radiolabeled peptide found in plasma and tissue extracts is determined according to the method of Thornburg *et al., (Am. J. Physiol.* 253:G68-G71 (1987)). Tissues are

homogenized in a total volume of 9 ml ice-cold distilled water using a Waring blender in a prechilled microjar. One milliliter of cold 0.5 N HCl is added and tubes are stoppered and hand mixed by inversion. Samples are kept on ice 30-60 minutes. After extraction and additional mixing, 0.1 or 1.0 ml aliquots of the homogenates are removed and counted in the gamma counter to determine total radioactivity. Samples are then transferred to 5/8 x 3 inch tubes and centrifuged at 100,000 x g for 40 minutes. Supernatant are decanted and aliquots are counted to determine extraction efficiencies. Approximately 90% of the homogenate radioactivity is extracted by this method. After rehomoginization of the pellet, greater than 95% of the radioactivity is extracted. Extracts are concentrated as required either by lyophilization or a Speed-Vac concentrator (Savant) and the residue is dissolved in 0.05 M phosphate buffer pH 7.4 and applied to a column.

Plasma is obtained from the supernatant after centrifugation (9,600 x g) of heparin-treated blood and analyzed directly. The pellet of blood cells is treated the same as other tissue samples.

Intact peptides are detected based on their appearance in the excluded void volume following Sephadex chromatography. The proportion of radioactivity in the void volume is calculated by summing the total cpm in this region and is expressed as a percent of the total radioactivity applied to the column. This percentage, along with the total radioactivities accumulated in specific tissues is used to calculate the total tissue recoveries of intact peptide. Results are expressed as a percentage of fed radioactivity.

Fractions containing intact or modified peptide are then further characterized by SDS gel autoradiography and reversed phase PHLC under dissociating conditions as detailed above.

B. The purpose of the following example is to determine the effect of the EGF-peptide or TFGα-peptide conjugate on the mitogenic activity of the epithelium.

Animals are fed peptides (80 ng to 1 μg) and simultaneously injected intraperitoneally with H-thymidine (1.5 μCi/gm body weight) for 2, 6, 12, 23 and 41 hours. Segments of proximal and distal intestine are rinsed with fixative *in situ* removed and immediately immersed in fixative. Tissues are chopped at 0.55 mm, dehydrated in graded alcohols and embedded in Epon-Araldite. Light microscopic autoradiography is then performed according to the following method. Sections (1μ) are mounted on glass slides and stained with iron-hematoxylin. Autoradiographs are prepared by coating slides with Ilford K5 photographic emulsion. The slides are kept refrigerated during exposure for 1 to 6 weeks, then developed. To quantitate epithelial proliferation, total epithelial cells and labeled epithelial cells are counted to determine the labelling index in 50 crypt regions in well-oriented sections. Cells are considered labeled if three or more exposed silver grains overlay the nucleus. Different sections from the same specimen are separated by at least ten serial sections to avoid scoring labeled cells more than once.

Migration rate of enterocytes is calculated by measuring the distance between the crypt base and the foremost labeled cell in 20 well-oriented crypt-villus units.

<u>We Claim</u>:

1. A method for delivery of a therapeutic agent to a subject comprising administering to the subject a chimeric molecule wherein said chimeric molecule comprises a therapeutic agent conjugated to a carrier wherein said carrier is capable of effecting delivery of said molecule by transepithelial transport via transcytosis.

2. The method of claim 1 wherein transcytosis is receptor-mediated.

3. The method of claim 1 or 2 wherein the chimeric molecule is administered orally and is absorbed from the gastrointestinal tract into the circulation.

4. The method of claim 2 wherein said receptor is the epidermal growth factor (EGF) receptor.

5. The method of claim 1 wherein said carrier is epidermal growth factor (EGF) or a receptor-binding fragment thereof or a receptor-binding analog thereof.

6. The method of claim 5 wherein said carrier is an EGF fragment containing amino acid residues 14-31 of mature EGF.

7. The method of claim 1 wherein said growth factor is transforming growth factor α (TGFα) or a receptor-binding fragment thereof or a receptor-binding analog thereof.

8. The method of claim 7 wherein said carrier is a TGFα fragment containing amino acid residues 34-43 of mature TGFα.

9. The method of claim 1 wherein said chimeric molecule is administered parenterally.

10. The method of claim 1 wherein said therapeutic agent is a drug.

11. The method of claim 1 wherein said therapeutic agent is a medicinal peptide.

12. A chimeric molecule comprising a therapeutic agent conjugated to a carrier, wherein said carrier is epidermal growth factor (EGF) or a receptor-binding fragment thereof or a receptor-binding analog thereof or wherein said carrier is transforming growth factor α (TGFα) or a receptor-binding fragment thereof or a receptor-binding analog thereof, and wherein said carrier is capable of effecting delivery of said molecule by transepithelial transport via transcytosis.

13. The chimeric molecule of claim 12 wherein said carrier is an EGF fragment containing amino acid residues 14-31 of mature EGF.

14. The chimeric molecule of claim 12 wherein said carrier is a TGFα fragment containing amino acid residues 34-43 of mature TGFα.

15. The chimeric molecule of claim 12 wherein said therapeutic agent is a drug.

16. The chimeric molecule of claim 12 wherein said therapeutic agent is a medicinal peptide.

17. The chimeric molecule of claim 12 wherein the therapeutic agent and carrier are conjugated via a conjugation agent.

18. The chimeric molecule of claim 17 wherein said conjugation agent is capable of conjugating the therapeutic agent and carrier by peptide thiolation or lysine coupling.

19. A composition comprising the chimeric molecule of claim 12 contained in a pharmaceutically acceptable solution or substance.

20. A method for delivery of a therapeutic agent into the circulation of a subject comprising orally administering to the subject a chimeric molecule wherein said chimeric molecule comprises a therapeutic agent conjugated to a carrier wherein said carrier comprises a growth factor or receptor-binding fragment thereof or a receptor-binding analog thereof and wherein said carrier is capable of effecting delivery of said molecule into said circulation via transcytosis.

21. The method of claim 20 wherein said carrier is or is derived from the epidermal growth factor (EGF).

22. The method of claim 21 wherein said carrier is an EGF fragment containing amino acid residues 14-31 of mature EGF.

23. The method of claim 20 wherein said carrier is or is derived from the transforming growth factor α (TGF-α).

24. The method of claim 23 wherein said carrier is a TGFα fragment containing amino acid residues 34-43 of mature TGFα.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/02874

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/02, 37/24, 37/36
US CL :514/12, 21
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12, 21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS EGF, FGF, conjugate, oral

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	US, A, 5,137,877 (Kaneko et al.) 11 August 1992, see the paragraph bridging columns 6 and 7, column 7, lines 49-68, column 19, lines 17-23.	1, 3, 5, 9-12, 15-21
Y	Proc. Natl. Acad. Sci., volume 81, issued March 1984, A. Komoriya et al. "Biologically Active Synthetic Fragments of Epidermal Growth Factor: Localization of a Major Receptor-Binding Region", pages 1351-1355, vol. 223, see the abstract.	1, 4, 5, 6, 10-13, 15, 16, 20-22

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 21 JUNE 1993	Date of mailing of the international search report 09 JUL 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer FATEMEH T. MOEZIE Fatemeh T. Moezie
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/02874

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, vol. 223, issued 09 March 1984, Hans Marquardt "Rat Transforming Growth Factor Type I: Structure and Relation to Epidermal Growth Factor" pages 1079-1082, see the abstract.	1, 7, 12, 14, 20, 23
A	The Journal of Biological Chemistry, Volume 258, No. 22, issued 25 November 1983, Joan Massague' "Epidermal Growth Factor-like Transforming Growth Factor" pages 13606-13613.	1-24